

A Monoclonal Antibody That Blocks the Activity of a Neurite Regeneration-Promoting Factor: Studies on the Binding Site and its Localization In Vivo

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Abstract. Work from several laboratories has identified a proteoglycan complex secreted by a variety of non-neuronal cells that can promote neurite regeneration when applied to the surface of culture dishes. Using a novel immunization protocol, a monoclonal antibody (INO) was produced that blocks the activity of this outgrowth-promoting factor (Matthew, W. D., and P. H. Patterson, 1983, *Cold Spring Harbor Symp. Quant. Biol.* 48:625–631). We have used the antibody to analyze the components of the active site and to localize the complex in vivo. INO binding is lost when the complex is dissociated; if its components are selectively reassociated, INO binds only to a complex containing two different molecular weight species. These are likely to be laminin and heparan sulfate proteoglycan, respectively. On frozen

sections of adult rat tissues, INO binding is present on the surfaces of glial cells of the peripheral, but not the central, nervous system. INO also binds to the basement membrane surrounding cardiac and skeletal muscle cells, and binding to the latter greatly increases after denervation. In the adrenal gland and kidney, INO selectively reacts with areas rich in basement membranes, staining a subset of structures that are immunoreactive for both laminin and heparan sulfate proteoglycan. In general, the outgrowth-blocking antibody binds to areas known to promote axonal regeneration and is absent from areas known to lack this ability. This suggests that this complex, which is active in culture, may be the physiological substrate supporting nerve regeneration in vivo.

AXONS damaged in the peripheral nervous system (PNS)¹ of adult mammals have the capacity to regenerate over long distances. The prerequisites for successful neurite outgrowth include the presence of trophic factors, such as nerve growth factor (3), and/or an appropriate surface on which to grow (30). The molecular nature of the surfaces that allow or stimulate regeneration in vivo is not known. Experiments in culture, however, have delineated a number of candidate molecules that can promote neurite outgrowth under specific conditions. For instance, surfaces coated with laminin or fibronectin stimulate a much more rapid and extensive regeneration of neuronal processes than can serum- or collagen-coated plastic (2, 4, 9, 24, 29, 31, 39, 44). The finding that laminin, in particular, provides an especially good substrate for outgrowth is of interest because a variety of non-neuronal cells secrete neurite regeneration-promoting factors (1, 12, 14, 26), some of which are extracellular matrix complexes containing primarily laminin and heparan sulfate proteoglycan (HeSPG) (8, 16, 28).

Since laminin is a component of a naturally occurring,

neurite-promoting complex, and purified laminin can stimulate outgrowth in culture, the question arises as to whether there is a role for the HeSPG component of the outgrowth factor. The complexity of the problem is indicated by the observation that antibodies that block the outgrowth-promoting effect of purified laminin do not usually block the effect of the natural complex (17, 27, 31). It is important, therefore, to understand the nature of the active site in the complex that stimulates neurite regeneration. To obtain information on the molecules that contribute to the active site, and to localize it in vivo, monoclonal antibodies were isolated that directly block the outgrowth-promoting activity of the complex (34). These antibodies were generated by a novel immunization procedure designed to produce monoclonal antibodies against rare antigens. The binding of one of these antibodies, INO (inhibitor of neurite outgrowth), to the complex requires the association of both laminin and HeSPG. We have also used this antibody to study the distribution of this epitope in vivo. The INO-binding site is found in areas known to promote axonal regeneration in vivo, and is not detected in areas where regeneration does not occur. These findings raise the possibility that the failure of central nervous system (CNS) axons to regenerate over long distances may be ex-

1. *Abbreviations used in this paper:* CM, conditioned medium; CNS, central nervous system; DRG, dorsal root ganglion; HeSPG, heparan sulfate proteoglycan; PNS, peripheral nervous system.

plained by the observed absence of this outgrowth-promoting complex.

Materials and Methods

Cell Culture and Conditioned Media

PC12 cells were maintained in L15 CO₂ medium (23), supplemented with 10% fetal calf serum and 5% heat-inactivated horse serum. Cells were grown in medium containing nerve growth factor and serum for 2 wk, then in serum-free medium for 12 h, rinsed, and fresh serum-free medium added. After 3 d, 500 ml of conditioned medium (CM) was collected and adsorbed to a 20-ml column of DEAE cellulose equilibrated with 150 mM NaCl and 10 mM phosphate (pH 7). The column was washed with 150 mM NaCl and the neurite outgrowth activity and HeSPG were eluted with 1.5 NaCl and stored at -20°.

A non-neuronal, fibroblast-like line established from primary dorsal root ganglion (DRG) cultures (33), secretes a high concentration of neurite-promoting activity. Cells were grown to confluence in L15CO₂ medium containing 10% fetal calf serum, then in serum-free medium for 2 d, rinsed, and maintained in a serum-free medium until the medium began to turn acidic (4-8 d). The final collection of 500 ml CM was then fractionated on DEAE cellulose as described above.

Fractionation of ³⁵S-Labeled Medium

The DRG non-neuronal cell line was grown to confluence in 75-cm² flasks in L15 CO₂ medium containing 10% fetal calf serum and then maintained for 2 wk in serum-free L15 CO₂ medium. Cultures were briefly rinsed in sulfate-free L15 CO₂ and each flask was incubated in 10 ml of medium containing 1 mCi of carrier-free [³⁵S]sulfate (ICN Biomedicals Inc., Irvine, CA). After 7 d, the CM was removed, stored on ice, and experiments were completed within 6 d. Approximately 1% of the radioactivity was excluded from Sephadex G25.

5 ml of labeled CM was incubated with 0.5 ml of DEAE (equilibrated with 0.15 M NaCl) for 30 min at room temperature. The DEAE was packed into a column and washed extensively with Hanks buffer (Gibco, Grand Island, NY) followed by 5 ml of calcium-magnesium-free Hanks buffer. The INO antigen was eluted with concentrated calcium-magnesium-free Hanks buffer (0.5 M NaCl). Fractions were collected, an aliquot of each was counted for ³⁵S, and appropriate samples were pooled. Where noted, solid guanidine HCl was added to bring an aliquot to a concentration of 6 M. These samples were stored overnight on ice before being chromatographed on a 1 cm × 26 cm column of Sepharose 4B. This column was either equilibrated with 0.15 M NaCl or 6 M guanidine. Single drops (50-μl each) were collected into individual wells of flexible microtiter dishes. Sequential samples were assayed with either a solid phase radioimmunoassay (25, 32) for binding of the INO antibody, pg42, or a control monoclonal antibody (SV 48) that recognizes a synaptic vesicle antigen and does not bind to CM, or counted for ³⁵S radioactivity. In those cases where samples contained guanidine, fractions were first pooled and then desalted on Sephadex G25 columns. Each value cited is the average of two samples. The void volume and the total volume for this column was determined using Blue Dextran and ³⁵S.

Antibodies

Monoclonal IgG antibodies C21 and C24, which recognize laminin (20); pg22, pg42 (33), and C17 (18), which bind to HeSPG; and monoclonal IgM antibody INO were obtained as previously described (33, 34, 42). Rabbit anti-rat N-CAM was provided by J. R. Sanes and J. Covault (Washington University, St. Louis, MO); rabbit anti-chicken neurofilament antibody (anti-NF160) was a gift of E. Lazarides (California Institute of Technology, Pasadena, CA). Rabbit anti-Po was supplied by G. Lemke (Salk Institute, La Jolla, CA) and J. Brockes (Medical Research Council, Cambridge, England); rabbit anti-mouse laminin antibody was purchased from Bethesda Research Laboratories (Gaithersburg, MD).

For the precipitation and competition assays, goat anti-mouse IgG, heavy- and light-chain specific, was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); biotinylated anti-mouse IgM, mu-chain specific, as well as avidin peroxidase, were supplied by Vector Laboratories (Burlingame, CA). For the immunofluorescence studies, high fluorescein-conjugated goat anti-mouse Ig was purchased from Antibodies Inc. (Davis, CA); rhodamine-conjugated goat anti-rabbit Ig was obtained from Cappel

Laboratories (West Chester, PA). Biotinylated goat anti-mouse IgM and fluoresceinated streptavidin were purchased from Cappel Laboratories and Bethesda Research Laboratories, respectively.

Precipitation Assay

Mouse ascites fluids, all containing at least 1 mg/ml monoclonal IgG antibody (pg22, pg42, C17, C21, C24), were diluted 1:200 in 100 mM Tris, (pH 7.2). Normal mouse serum and rabbit anti-mouse laminin serum (Bethesda Research Laboratories) were also used at 1:200 dilutions. All operations were carried out at room temperature. Individual wells of 96-well flat-bottom, Immunolon 1 plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were incubated with 40 μl of diluted antibody for 30 min, then incubated with 2% BSA in 100 mM Tris buffer for 10 min followed by four washes with Tris buffer. Washing consisted of adding 100 μl of buffer to each well, emptying the plate by inversion, and blotting on paper towels. Wells were next incubated for 1 h with fresh L15 CO₂ medium (control) or with serum-containing CM from either the DRG cell line or PC12 cells. The wells were washed four times and then incubated with 50 μl of fresh INO hybridoma medium for 2 h, followed again by four washes. The wells were incubated with 50 μl of 1% BSA containing 20 μg/ml goat anti-mouse IgG for 30 min to help prevent cross reaction with subsequent anti-mouse IgM and the ascites IgG. Without washing, 50 μl of biotinylated anti-mouse IgM (10 μg/ml) antiserum was added for a 2-h incubation. The wells were washed four times before adding 100 μl of avidin-peroxidase reagent for 1 h. After washing, peroxidase was reacted with diaminobenzidine and 0.03% peroxide in Tris. The reaction was stopped after 5 min by addition of 50 μl of 1% HCl and the reaction product was measured as OD at 405 nm.

Competition Assay

Wells were coated with 100 μl of serially diluted, serum-free CM from either the DRG or PC12 cell line. A serial dilution of INO culture fluid in 1% BSA-Tris buffer (pH 7.2) was also bound to the wells to determine the appropriate concentrations of CM and INO culture fluid such that the amount of INO antibody would be limiting in each assay. A 1:150 dilution of INO culture fluid was found to give a signal of 65% of the maximum signal for a 1:4 dilution of PC12 CM, and 80% of maximal signal for a 1:256 dilution of DRG cell CM. All subsequent assays used the same stock of CM (1:4 for PC12 and 1:256 for DRG cells) and a stock of 1:15 dilution of INO culture medium stored at 4°C for not more than 3 d.

Immunolon wells were coated with fresh L15 CO₂ medium (control) or with either DRG or PC12 cell CM for 30 min and washed. Wells were then incubated for 1 h at room temperature with 50 μl of competing antibody: ascites fluids were all used at >1 mg/ml monoclonal IgG antibody while normal mouse serum and rabbit anti-mouse laminin serum were diluted 1:200 with 2% BSA-Tris. Without washing, 50 μl of a 1:75 dilution of INO culture fluid was added to each well and co-incubated for 1 h before washing. The specific binding of mouse IgM (INO) was subsequently quantified as described for the precipitation assay.

Immunohistochemistry

Freshly dissected, unfixed rat tissues were mounted on metal chucks with gum tragacanth (Eastman Kodak Co., Rochester, NY) and flash frozen in liquid nitrogen-cooled isopentane. For direct comparisons of staining patterns and intensities, several tissue types were assembled, frozen, sectioned, stained, and photographed together. Sections (4-10 μm) were cut on a cryostat (Hacker Instruments Inc., Fairfield, NJ), air dried on ethanol-cleaned glass slides and stored frozen until used.

After a 10-min preincubation at room temperature in PBS containing 10 mg/ml BSA (PBS-BSA), sections were incubated overnight with primary antibody at 4°C in a humidified chamber. INO hybridoma medium was used undiluted; C17, C21, and C24 ascites fluids were diluted to 1:250. In double-label experiments, sections were co-incubated with a mouse monoclonal and a rabbit antiserum. Anti-N-CAM was used at a 1:200 dilution; anti-NF160 and anti-Po were diluted to 1:100. All dilutions were made with PBS-BSA. Excess primary antibodies were removed by rinsing the slides thrice with PBS (10 min each), and nonspecific binding sites were blocked with a 10-min incubation in PBS-BSA containing 10% normal goat serum before applying marker-conjugated secondary antibodies.

Because mouse and rat immunoglobulins share many determinants, secondary antibodies against mouse immunoglobulins were diluted and preabsorbed with PBS-BSA containing 10% normal rat serum for 1 h at room temperature, and centrifuged in an Eppendorf microfuge for 10 min before

use. This serves to remove the subset of secondary antibodies that also recognize rat determinants on the sections, significantly reducing background staining by secondary antibodies. Fluoresceinated goat anti-mouse Ig(1:100 dilution) was used to visualize C17, C21, and C24 binding; rhodaminated anti-rabbit Ig (1:100) revealed binding by antisera. To view INO immunoreactivity, a sandwich consisting of biotinylated goat anti-mouse IgM (1:75 dilution) followed by fluorescein-streptavidin (1:50) was used. Sections were incubated with secondary and then tertiary reagents for 1 h each at room temperature before mounting with glycerol-phenylenediamine (46) for viewing. To identify nuclei, a final pulse (5–10 s) of 10 µg/ml propidium iodide was applied and rinsed just before mounting. Propidium iodide binds to nucleic acids and can be viewed with filters for rhodamine.

Endplate-bearing sections (2 µm) of diaphragm were obtained as described by Sanes and Hall (41) and synaptic sites identified by co-incubation with rhodaminated- α -bungarotoxin (38), which binds to acetylcholine receptors on the postsynaptic membrane. For studies on denervated muscle, the left phrenic nerve of adult (>150 g) female rats was cut under ether anesthesia, and a 2–4-mm segment removed to prevent reinnervation. After appropriate intervals (4 d to 4.5 wk), the animals were sacrificed. Denervated diaphragm was sandwiched between pieces of the contralateral, innervated control hemidiaphragm and sectioned for immunohistochemistry. Empty sheaths of muscle basal lamina were obtained by surgically inducing degeneration of intercostal myofibers (39); 3–4 d later the damaged tissue was dissected and sectioned.

Results

Composition of the INO-binding Site

The source for the neurite-promoting activity used in this study was a non-neuronal cell line isolated from a primary culture of newborn rat DRG cells (33). This line secretes a high level of the INO antigen as well as the neurite-promoting activity. Five monoclonal antibodies that recognize determinants on HeSPG and precipitate the neurite-promoting activity in CM, bind to the surface of these cells in culture (33). These DRG-derived cells were incubated with ^{35}S -labeled sulfate, a procedure that primarily labels proteoglycans (22, 35). The INO antigen was coeluted from DEAE cellulose between 0.3 and 0.5 M NaCl and chromatographed on Sepharose 4B. Both the pg42 (a monoclonal antibody that binds the core protein of HeSPG [33] and can precipitate the neurite promoting activity but not block it directly) and INO immunoreactivity coeluted with most of

the ^{35}S -labeled material, which had an apparent molecular mass of $\sim 10^7$ D (Fig. 1). Thus neurite-promoting activity, as identified by INO binding, is associated with a HeSPG. To determine whether the INO-binding site is part of the HeSPG itself or is associated with a complex of HeSPG with another molecule, the sulfate-labeled DEAE fraction was dissociated in 6 M guanidine and chromatographed on Sepharose 4B equilibrated with 6 M guanidine (Fig. 2). Sulfate label and HeSPG antigen (as measured by pg42 binding) migrated with a molecular mass of ~ 300 kD. No INO binding could be detected after guanidine removal (Fig. 2). Therefore, either the antigen was destroyed by guanidine or the determinant results from the association of several different molecules.

The latter possibility was tested in a reassociation experiment. CM was fractionated in guanidine over a Sepharose column as before, and fractions were pooled into four large groups (Fig. 3). Only one fraction, C, contained HeSPG (Table I). The four fractions were recombined in all combinations of two, the guanidine removed, and each combination was tested for INO antigenicity. Most of the INO binding was obtained by mixing the HeSPG fraction with a high molecular weight fraction that does not contain a significant amount of sulfate label or HeSPG antigen, but which very likely does contain laminin because of laminin's very high molecular weight (Table I).

These results suggested that the INO epitope is associated with HeSPG and laminin. In fact, several groups have provided convincing evidence that the outgrowth-promoting activity is associated with a HeSPG–laminin complex (16, 28). To confirm that this was also true in the CM used in the present study, several monoclonal and polyclonal antibodies to laminin and HeSPG were tested for their ability to precipitate the INO determinant from the DRG cell line CM and PC12 cell CM (Table II). Both sets of antibodies were able to precipitate significant amounts of INO antigen, whereas a control antibody, SV48, did not.

As an additional test for the contribution of laminin and HeSPG to the INO determinant, monoclonal and polyclonal antibodies against laminin and HeSPG were used to compete

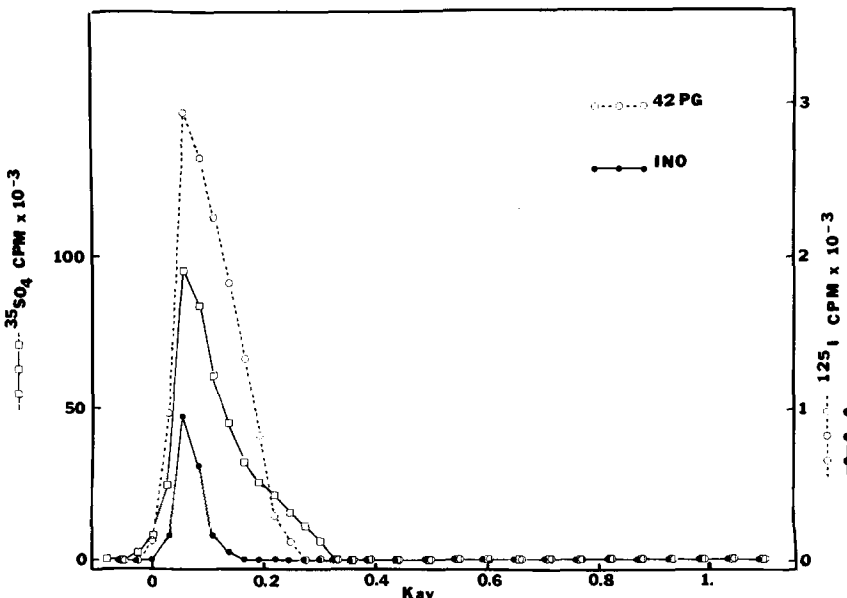


Figure 1. Sepharose 4B column fractionation, under native conditions, of ^{35}S -labeled CM from a DRG non-neuronal cell line. Labeled medium was prepared and fractionated as described in Materials and Methods. Fractions were analyzed for ^{35}S radioactivity (\square), and assayed for the presence of INO (\bullet) and HeSPG (\circ) immunoreactivity, using the INO and pg42 antibodies, respectively. K_{av} (elution volume – void volume) \div (total volume – void volume).

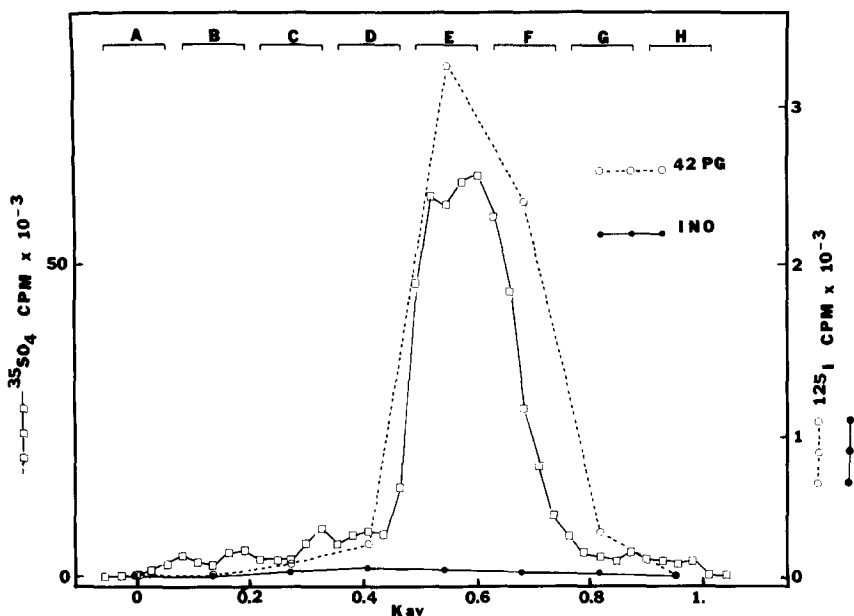


Figure 2. Sepharose 4B column fractionation, under denaturing conditions, of DRG cell CM. Labeled CM was prepared as described in Materials and Methods, adjusted to 6 M guanidine, and fractionated in 6 M guanidine. Every third fraction was counted for radioactivity to determine the glycosaminoglycan profile. Pools A-H were made from the remaining fractions, desalted, and assayed for immunoreactivity with the pg 42 and INO antibodies. Fractions were analyzed for ^{35}S radioactivity (\square), and assayed for the presence of INO (\bullet) and HeSPG (\circ) immunoreactivity, using the INO and pg42 antibodies, respectively. K_{av} , (elution volume - void volume) \div (total volume - void volume).

with the INO antibody for its binding site. It is clear that both sets of antibodies are able to effectively inhibit the INO antibody in its binding to CM from two sources (Table III). Therefore, several lines of evidence support the idea that INO binds to a high molecular weight acidic complex made up of at least two molecules, most likely laminin and HeSPG. It is interesting that the HeSPG antibodies were generally better than the laminin antibodies both at precipitating the INO-bearing complex and inhibiting INO binding to the complex.

Localization of INO Immunoreactivity in the Adult Rat Nervous System

We used direct immunofluorescence to study the distribution of INO binding in the PNS and CNS of the adult rat. INO stained the surfaces of non-neuronal cells (Schwann cells and satellite cells) in all parts of the PNS examined. Within the DRG and the superior cervical ganglion, INO binds to the basement membrane of satellite cells that ensheaths neuronal cell bodies and processes (Figs. 4 and 5). Non-neuronal cells elaborate an extensive extracellular matrix within peripheral ganglia, especially the SCG; the pattern of INO staining

strongly resembles patterns seen with antibodies to known matrix components including laminin (using C21 and C24) and HeSPG (using C17). In the sciatic nerve, a mixed peripheral nerve trunk, INO binds to the outer or abaxonal surface of both myelinating and unmyelinating Schwann cells, although the antibody appears to stain the sheath around un-

Table I. Reconstitution of the INO Determinant from Guanidine Extracts

Fraction	pg 42 in radio-immunoassay cpm	INO in radio-immunoassay cpm
A	136	122
B	174	199
C	5,877	136
D	161	85
A+B	150	246
A+C	4,992	190
A+D	123	150
B+C	3,281	1,869
B+D	145	427
C+D	4,048	311

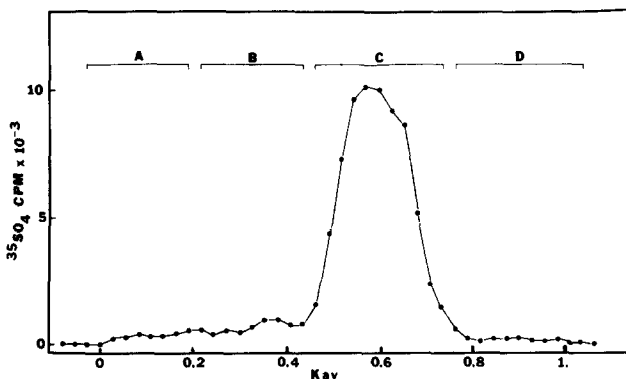


Figure 3. Sepharose fractionation of CM under denaturing conditions for a reassociation experiment. CM was produced and fractionated as described for Fig. 2 except that fractions A-D were pooled as shown.

Table II. Monoclonal and Polyclonal Antibodies to Laminin and HeSPG Can Precipitate the INO Antigen

Precipitating antibody	DRG cell CM $OD_{405} \times 10^3$	PC12 cell CM $OD_{405} \times 10^3$
HeS pg22	2,397	1,913
HeS pg42	2,447	1,926
HeS C17	1,080	530
Lam C21	1,437	624
Lam C24	1,180	642
Anti-laminin	1,786	1,192
SV 48	7	38

Precipitating antibodies were absorbed to 96-well immunoplates, which were then washed and incubated with CM. After washing, INO antibody was incubated and immunoreactivity was detected using an enzyme-linked immunosorbent assay.

Table III. Monoclonal and Polyclonal Antibodies to Laminin and HeSPG Can Compete with the INO Antibody for Binding to CM

Competing antibody	DRG cell CM (% inhibition)	PC12 cell CM (% inhibition)
BSA control	0	0
HeS pg22	81	90
HeS pg42	85	90
HeS C17	46	49
Lam C21	10	22
Lam C24	11	23
Anti-laminin	82	79
SV 48	3	14

CM were absorbed to 96-well immunoplates, which were then washed and incubated with competing antibodies. A limiting amount of INO antibody was added and INO immunoreactivity was detected using an enzyme-linked immunosorbent assay.

myelinated fibers much more brightly (Fig. 6). Double-label experiments, using INO and anti-NF160 to mark the location of axons, demonstrate that the side of the ensheathing Schwann cell that faces axonal surfaces is devoid of immunoreactivity, as is the axon. Because only the abaxonal Schwann cell surface is known to organize and accumulate a basement membrane, this asymmetry in INO immunoreactivity supports the notion that the antibody recognizes a component within the basement membrane or the extracellular matrix of the endoneurium. The mAb's to HeSPG and to laminin (C17, C21, and C24) similarly stain the endoneurium in peripheral nerves (18, 42).

Unlike C17 and C24, INO and C21 do not stain the loose connective tissue, or epineurium, which surrounds peripheral ganglia (Fig. 5) and major nerve trunks. INO, C17, and C24, but not C21, also bind to the perineurium that groups fascicles of nerves and forms the outer connective tissue sheath around small intramuscular nerves when they split off from major nerve trunks (Fig. 7). INO immunoreactivity continues down nerves to their terminal arborizations in muscle.

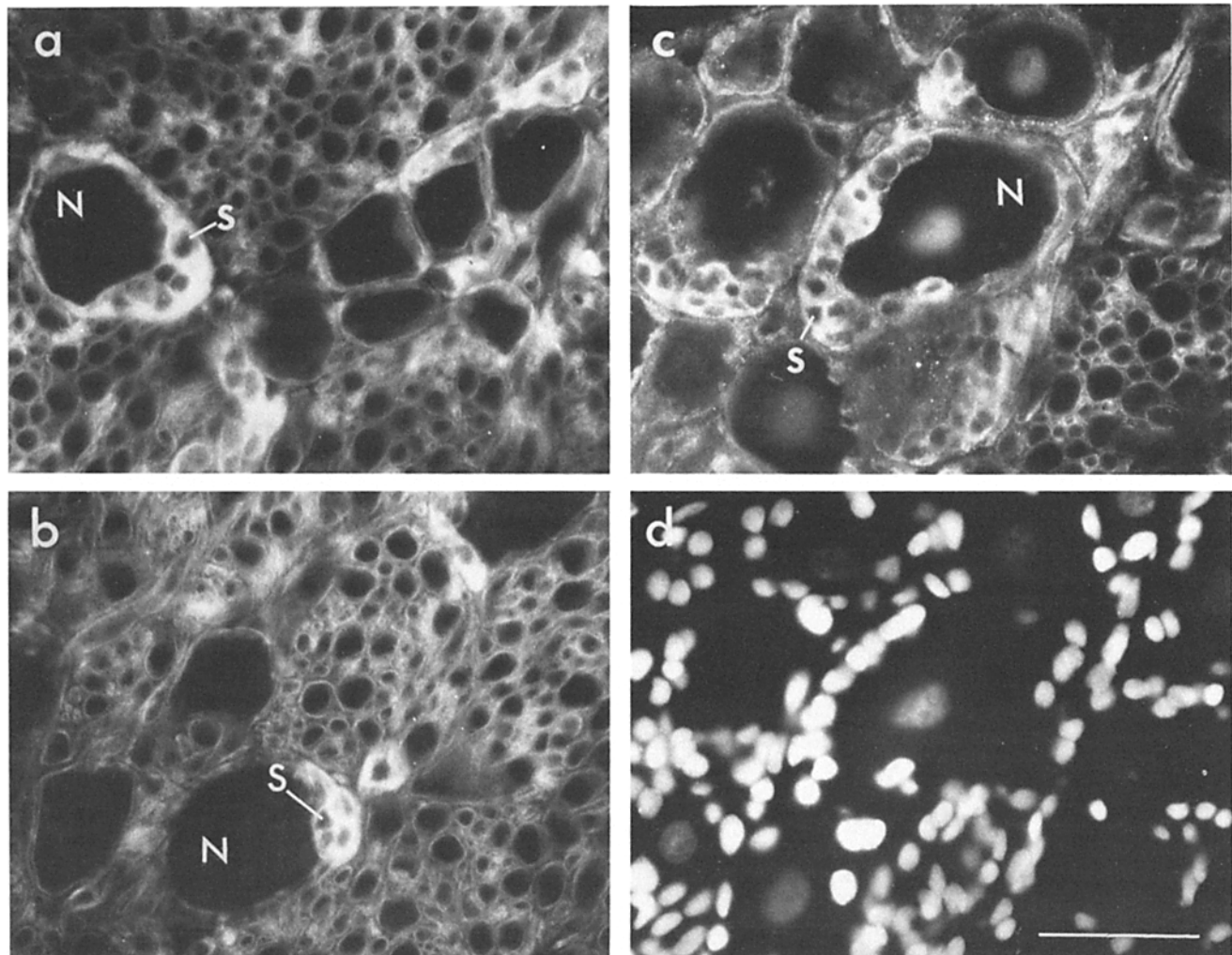


Figure 4. Endoneurium in adult rat DRG bears INO, laminin, and HeSPG determinants. Antigens recognized by C17 (a), C24 (b), and INO (c) are present on the surface of satellite cells (s), some of which cluster along the periphery of the sensory neurons (N). The INO-stained section (c) was double-labeled with propidium iodide (d) to reveal nuclei of neurons and satellite cells. Whereas INO clearly stains a unique epitope in neuronal nuclei, antibody binding is not detectable in nuclei of satellite cells. Bar, 50 μ m.

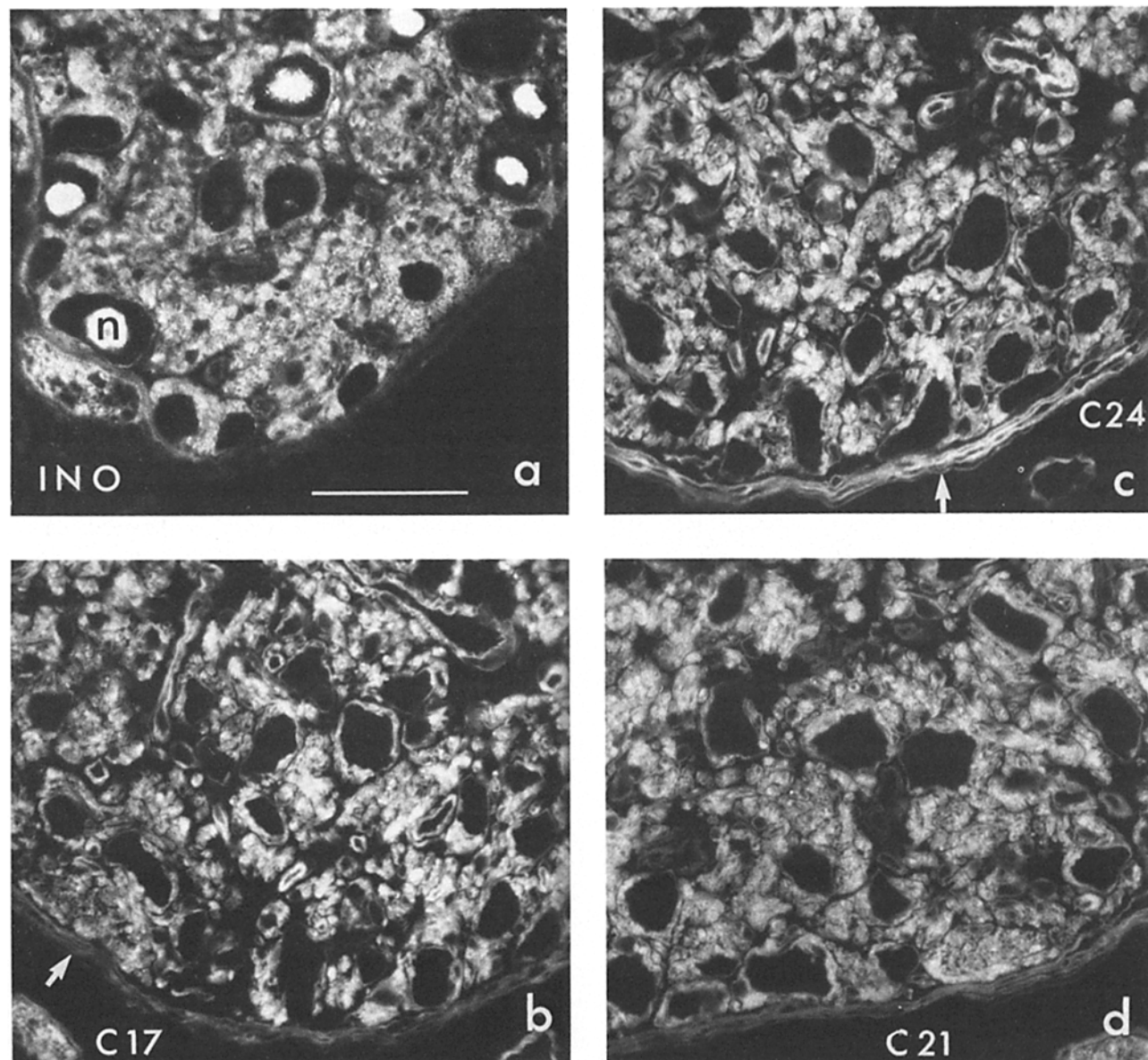


Figure 5. Immunohistochemical staining of 10- μ m sections of rat superior cervical ganglion reveals similar patterns of cell-surface staining with INO (a), C17 (b), C24 (c), and C21 (d). All four antibodies recognize components that appear abundant in the extracellular matrix on the surfaces of satellite cells ensheathing the neurons. Only INO stains neuronal nuclei (*n* in a). Whereas C17 and C24 also bind to the epineurium (arrow), INO and C21 do not. Bar, 50 μ m.

Peri- and endoneurial staining by INO is present in both dorsal and ventral spinal roots; thus both proximal and distal projections of peripheral nerves maintain immunoreactivity. However, sections of spinal cord reveal that staining by INO, C17, C21, and C24 ceases at the transitional region (6) between the PNS and the CNS (Figs. 8 and 9), and, except for the binding of capillary and meningeal surfaces, there is no detectable antibody binding within either grey or white matter of the spinal cord. An exception is the binding of INO to neuronal nuclei, which is discussed below. Similarly, sections of optic nerve and of olfactory bulb are devoid of staining (not shown), whereas flanking sections of sciatic nerve are highly immunoreactive. Because blood vessels and meningeal membranes are the only places within the CNS where an organized basement membrane is visible ultrastructurally, the absence of antibody binding is consistent

with the localization/restriction of INO-, C17-, C21-, and C24-binding sites to the extracellular matrix. The restriction, therefore, of INO binding to the PNS may be a consequence of the lack of an organized basement membrane within the adult CNS.

Distribution of INO Binding in Muscle and Other Basement Membranes

The cell surface INO epitope is also present on surfaces of three muscle cell types examined. Sections of rat ventricle and diaphragm show that both cardiac and skeletal muscle fibers are stained by INO (Fig. 10 a). In sections of muscle spindles, INO binds to the capsule as well as to both bag and chain intrafusal fibers (Fig. 10 b). Immunoreactivity on skeletal muscle extends to the very ends of the fibers where

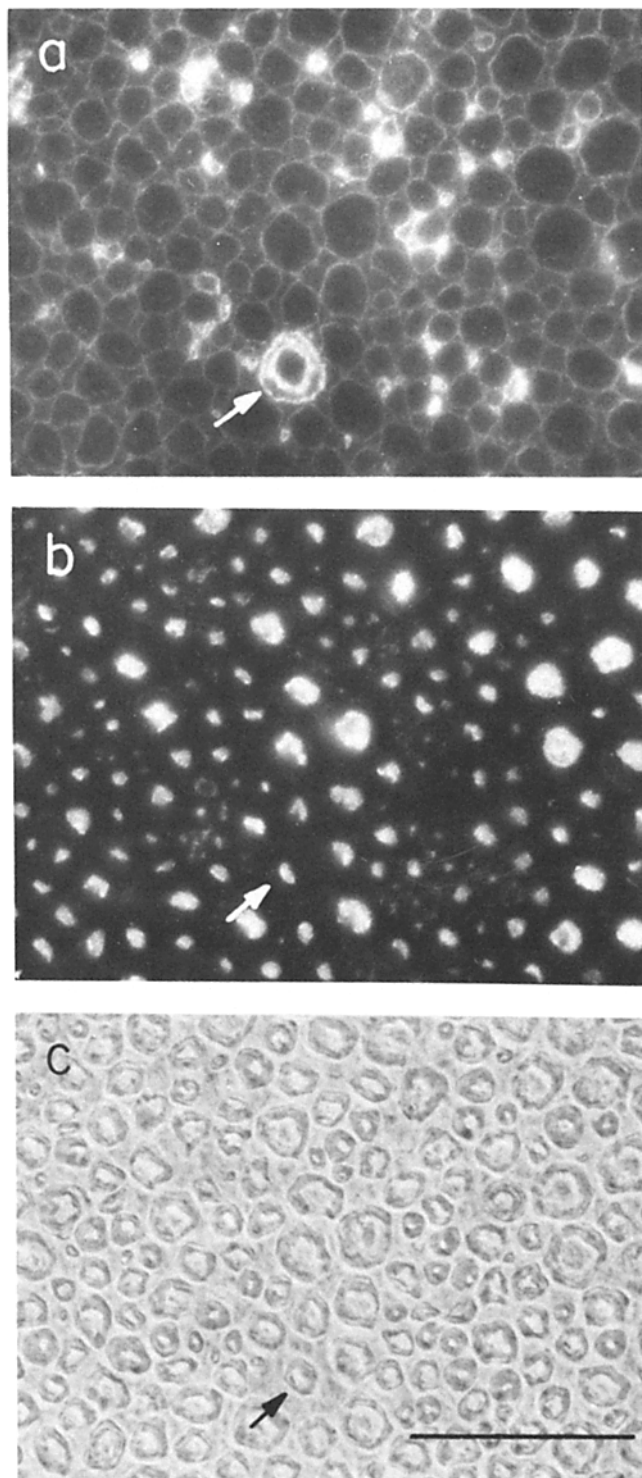


Figure 6. Sections of rat sciatic nerve stained with INO (*a*) and anti-NF 160 (*b*) show INO reactivity is restricted to the abaxonal surfaces of Schwann cells. The neurofilament-containing profiles are clearly not in contact with the INO sites, both on myelinated and unmyelinated fibers. Only the abaxonal surface is stained by C17, C21, and C24 (data not shown) and bears a basal lamina. In the bright, triple-laminated profile (*arrow*), the axon appears to be enclosed by a myelinated sheath, which in turn has been wrapped by a second Schwann cell. Thus the abaxonal basal lamina of the myelinating Schwann cell faces another Schwann cell and bears a basal lamina on both surfaces. Such profiles are rare. Bar, 50 μ m.

myotendinous junctions are found to be brightly stained (not shown). When skeletal myofibers were damaged to generate empty basal lamina sheaths, INO staining persisted in these preparations and co-localized with profiles concomitantly stained with anti-laminin, which marked the empty tubes of basal lamina (Fig. 10, *c* and *d*). In skeletal muscle, INO is also present at the neuromuscular junction where it co-localizes with rhodamine- α -bungarotoxin, a marker for post-synaptic acetylcholine receptors (Fig. 10, *e-h*). Thus in muscle, as in nerve, INO appears to recognize an epitope present throughout the muscle basement membrane.

Molecules that are involved in neuronal adhesion (15) or in the promotion of neurite outgrowth (21, 45) have been found to increase when muscles are denervated. To examine the effects of denervation on the amount or distribution of INO, innervated and denervated hemidiaphragms were mounted in a single block and then sectioned, stained, and photographed together. As it is known that levels of N-CAM rise in denervated muscle, sections were doubly labeled with anti-N-CAM to confirm successful denervation. We found that a rise in INO immunoreactivity was detectable on the surfaces of muscles after 1 wk of denervation; this difference in staining intensity between innervated and denervated muscles increased with time and was quite dramatic by 4.5 wk (Fig. 11). Although smaller, an increase was also detected with C17, the monoclonal antibody that recognizes HeSPG. However, no difference between innervated and denervated muscle was detectable when consecutive sections were reacted with C21, C24, or polyclonal antibodies to laminin, which suggests that the increase in INO and C17 staining intensity is not merely due to a general increase in basal lamina, or to muscle atrophy. Similar results were reported by Sanes and co-workers (43), who, using antibodies against laminin and fibronectin, also detected no change in the staining of basal lamina of muscle after denervation. However, in contrast to our results with C17, they did not see any consistent increase in levels of HeSPG upon denervation. If suprathreshold levels of primary and secondary antibodies were used in these comparative studies, such differences in staining intensity may be less apparent. We find a specific rise in the INO epitope concurrent with an increase in C17 binding, in the apparent absence of any change in laminin content or distribution, when muscles are denervated.

In both muscle and nerve, INO binds to areas rich in basement membranes; however not all basement membranes bear this epitope. We analyzed INO binding in two other tissues known to be rich in extracellular matrix components (Table IV). Within the adult rat adrenal gland, INO binds to connective tissue that surrounds groups of chromaffin cells in the adrenal medulla. Double-staining with anti-NF160 indicated that large bundles of innervating sympathetic axons travel along these corridors of extracellular matrix (not shown). Staining was absent in the adrenal cortex. This distribution precisely matches that of laminin; however HeSPG is present in both medullary and cortical areas of the adrenals. The kidney, a tissue rich in basement membranes, was heavily invested with HeSPG and laminin. INO, however, shows a very restricted distribution and could be detected only on the glomerular capsule. Similarly, INO did not bind to major blood vessels, which bear a basal lamina rich in HeSPG and laminin (Fig. 7).

In summary, INO binds to basal lamina-rich regions in

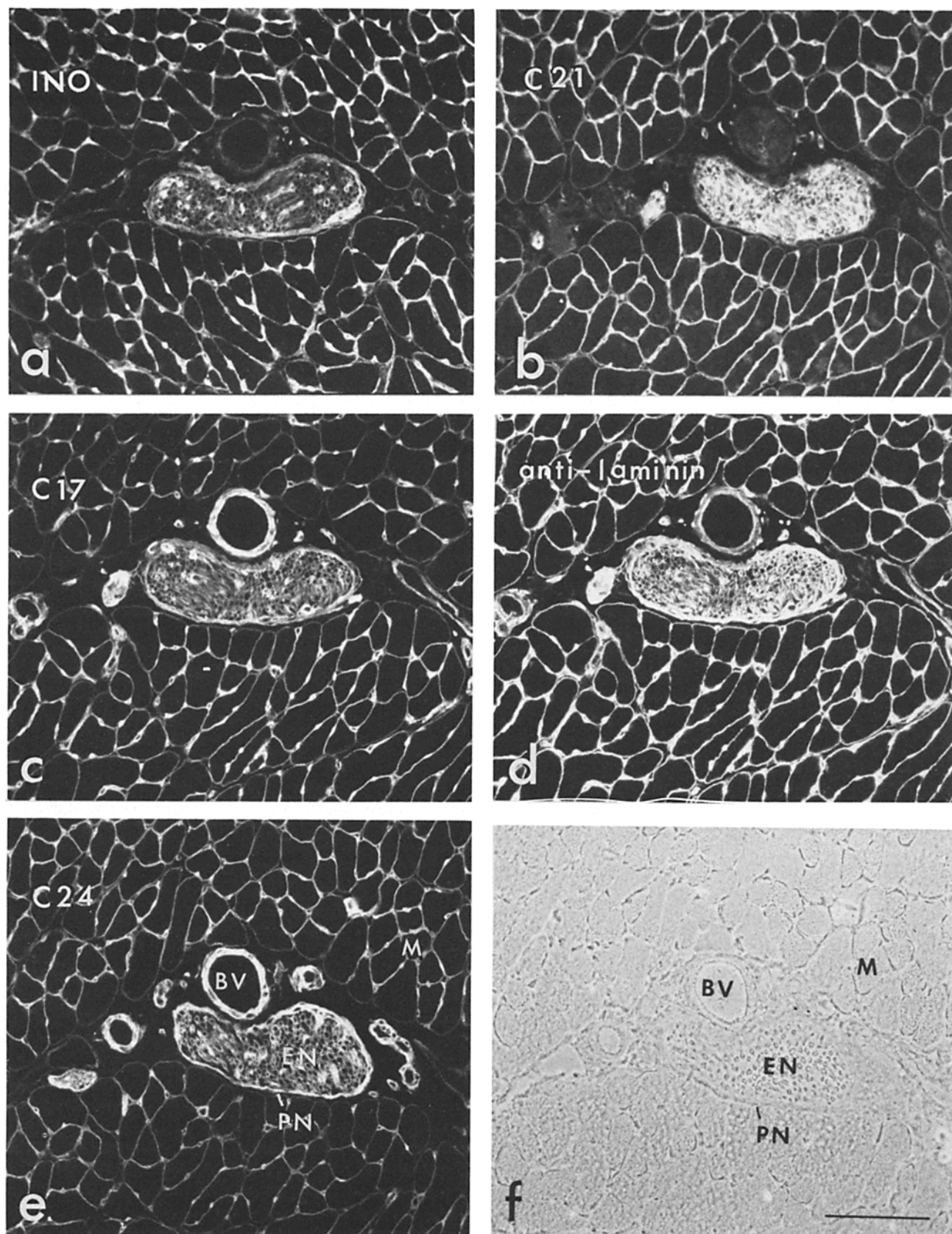


Figure 7. Consecutive sections of rat diaphragm, stained with INO (*a*), C21 (*b*), C17 (*c*), anti-laminin (*d*), and C24 (*e*) demonstrate that INO binds to a subset of basement membranes that bear both HeSPG and laminin antigens. Four types of basement membranes are present in each field: skeletal muscle basal lamina (*M*), blood vessel basal lamina (*BV*), endoneurium (*EN*), and perineurium (*PN*) of intramuscular nerves. Anti-laminin, C17, and C24 stain all four types of basement membranes; INO immunoreactivity is present in all except blood vessels. C21, which recognizes a more restricted laminin epitope, is absent in both blood vessels and perineurium. Thus, not all laminin epitopes are present or available for binding in laminin-bearing matrices, and INO binding is detectable in some but not all structures bearing both HeSPG and laminin. (*c* and *d*) The same field double-labeled with C17 and anti-laminin *f* is the phase view of the field stained with C24 in *e*. Bar, 100 μ m.

nerve, muscle, and adrenal medulla that normally can support neurite outgrowth. The enrichment of the INO epitope on denervated muscle raises the possibility that this epitope may be involved in the propensity of denervated muscle to promote neuronal sprouting and reinnervation. The distribution of the INO epitope in other basement membranes is, however, much more restricted. Whereas all INO binding sites in the extracellular matrix also contain HeSPG and some form of laminin, not all basement membranes containing both of these matrix components show INO immunoreactivity. The manner of their association, as well as the specific isoforms of these two molecules (see Discussion), may determine whether the INO determinant is expressed. Thus, HeSPG and laminin appear to be necessary, but not sufficient, for the generation of the INO epitope in extracellular matrices.

As reported previously (32), INO also recognizes an epitope in adult neuronal nuclei (Figs. 4, 5, and 9) and in nuclei undergoing active transcription, such as in liver, epithelia, spermatogonia (but not sperm), and developing (but not adult) and regenerating muscles. While this is intriguing, it is unlikely that this epitope resides in the same complex as the neurite-promoting factor. None of our antibodies to HeSPG or to laminin bind to nuclei; thus if these molecules are found in the nucleus (19), they must be different from those in the extracellular matrix. Although monoclonal antibodies are described as monospecific in that they recognize a very defined determinant, they cannot discriminate between different molecules which happen to share the same epitope. It is therefore likely that the nuclear-binding site for INO resides in a molecule(s) different from the neurite-promoting complex in the extracellular matrix.

Discussion

The ability of purified laminin to support and guide neurite regeneration *in vitro* has raised the possibility that the laminin component of the native complex is solely responsible for the outgrowth activity in CM. This simple interpretation has been challenged by the observation that antibodies to laminin, which block laminin-induced outgrowth, fail to abrogate the activity of the naturally secreted factors (8, 15, 25, 29). Thus it is unclear what comprises the neurite-promoting site on the native complex, nor is it known what is the physiological substrate on which axons regenerate *in vivo*. Since the monoclonal antibody INO can directly block neurite regeneration stimulated by at least two naturally occurring factors (34), understanding the requirements for and the distribution of INO binding should yield information on the native neurite-promoting site.

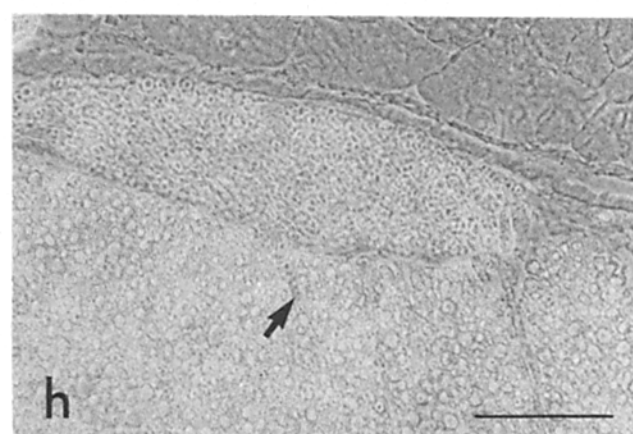
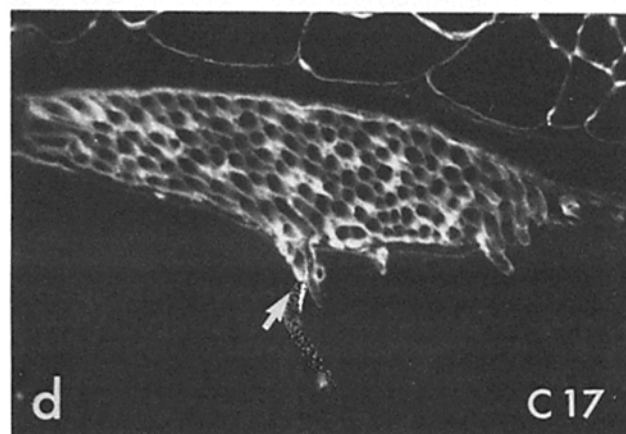
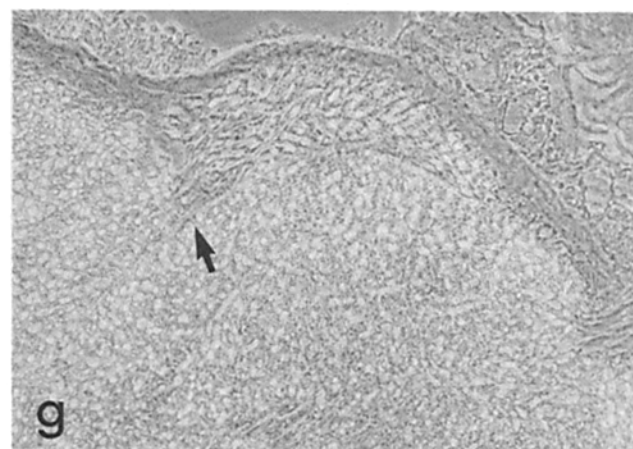
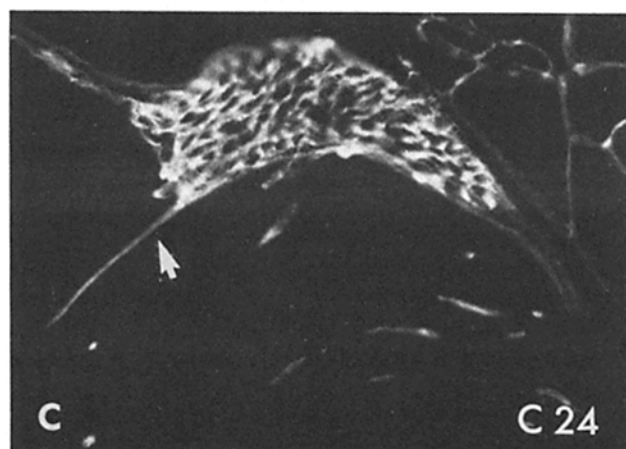
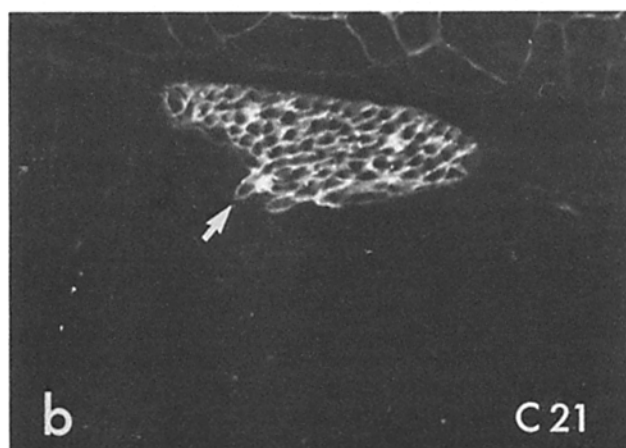
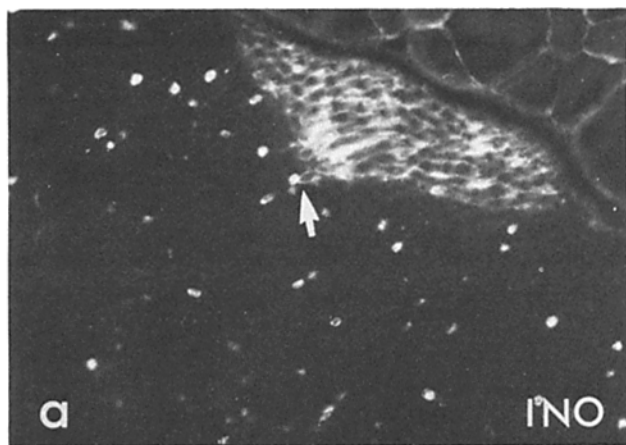
INO appears to recognize an epitope generated by the association of at least two components, most likely laminin and HeSPG. Polyclonal and monoclonal antibodies specific for laminin or for HeSPG selectively precipitate the INO-bearing complex harvested from media conditioned by the DRG cell line and by PC12 cells. Other investigators have also found that neurite-promoting factors released by a variety of cells in culture, including chick skeletal muscle (8), rat Schwannomas (16), and bovine corneal endothelial cells (28), consistently contain both laminin and HeSPG. INO binding is lost when the complex is dissociated and fractionated into its components; however, immunoreactivity can be recovered upon selective reassociation of two of these frac-

tions. This data shows that neurite-promoting activity of the natural laminin-HeSPG complex is amenable to direct antibody blockade only when its components are in association.

These results raise the questions of how formation of the complex results in the generation of the INO-binding site, and why anti-laminin antibodies are unable to block the activity of the native complex. In studies of different binding domains on the laminin molecule, only antibodies directed against the major HeSPG-binding fragment are competent to block the neurite-promoting activity of laminin (17). This supports the idea that the neurite-promoting site on laminin, site A, is located at or near where HeSPG binds, and anti-laminin sera presumably block bioactivity by binding at or near this site. Formation of the HeSPG-laminin complex could have several possible outcomes. First, the proteoglycan may convert site A to a new active form, B, which is now recognized by INO but not by blocking anti-laminin antibodies. Heparan sulfates have been reported to induce conformational changes in fibronectin, another component of the extracellular matrix (36). Alternatively, association with HeSPG may sterically hinder access of the relevant anti-laminin antibodies and at the same time create a new epitope, the INO-binding site, nearby so that INO binding now blocks the availability/activity of site A. Interestingly, neurons are known to bear a HeSPG on their surfaces (33), and interactions between HeS and N-CAM appear to influence the homophilic binding of N-CAM during neuron-neuron adhesion (10, 11). During the outgrowth of processes, it is possible that neurons form their own HeSPG-laminin association by displacing the HeS in the complex with their own HeS moiety, thereby promoting neuritic extension. The binding of INO may prevent this neuron-matrix association and so curtail outgrowth. Thus, HeSPG on cell surfaces may regulate interactions between neurons and the environment.

Some investigators have proposed that the inability of anti-laminin to block regeneration stimulated by the complex can be explained by species and tissue differences in laminin isoforms. Most investigators use laminin purified from the mouse Englebreth-Holm-Swarm sarcoma, and anti-laminin antisera is most commonly generated using this form of laminin as the immunogen. While it is likely that rat and mouse laminin share a fair degree of homology, it is still possible that sufficient heterogeneity exists at the neurite-promoting site such that antibodies to mouse laminin are unable to block the activity of rat laminin. However, antibodies generated against rat yolk sac laminin block neurite promotion by both rat and mouse laminin, yet proved inadequate in preventing the action of the proteoglycan-laminin factor secreted by rat Schwannomas (31). Results such as these have led to the suggestion that different tissues may produce different forms of laminin, even within the same species. Indirect support for this idea comes from immunofluorescence studies showing non-identical distribution of different laminin epitopes within the same animal (20, 47). Nevertheless, it appears improbable that while laminin from mouse Englebreth-Holm-Swarm sarcoma and rat yolk sac bear similar bioactive sites, the outgrowth-promoting active site on laminin produced by a rat cell line would be radically different. A more likely explanation may be that the regeneration-promoting active site on the rat Schwannoma complex is not recognized by or is not accessible to blocking antibodies which only recognize laminin.

Comparative immunofluorescence studies with INO and



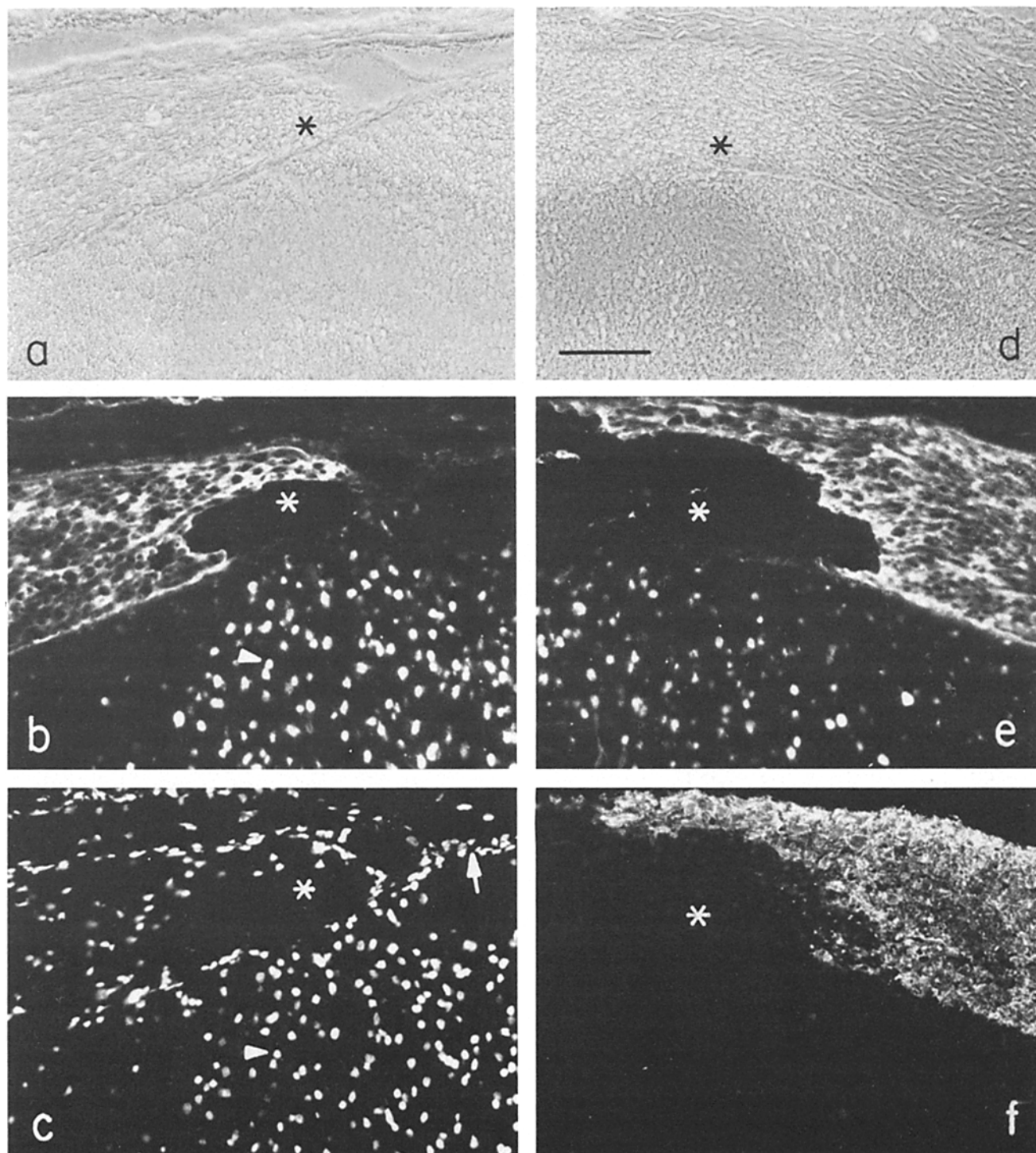


Figure 9. Immunoreactivity for INO and for Po ends at the transitional zone between the PNS and the CNS. In sections of spinal cord where the CNS protrudes, dome-like (asterisk) into the proximal end of the dorsal root, double-labeling with INO (*e*) and with anti-Po (*f*) shows that binding sites for both antibodies end abruptly at the transitional zone. A consecutive section (*a-c*), which was double-labeled with INO (*b*) and with propidium iodide (*c*) to localize nuclei, shows that INO stains nuclei, presumably of neurons in the dorsal horn (arrowhead) while glial nuclei (arrow) present outside of the horn area are not immunoreactive. The dome-shaped transitional zone also bears few stained nuclei. Phase micrograph of the field in *b* and *c* is shown in *a*, and of *e* and *f* in *d*. Bar, 100 μ m.

Figure 8. Immunofluorescent demonstration of the presence of the INO, laminin, and HeSPG antigens in the PNS but not in the CNS. 10- μ m sections of adult rat spinal cord show only ventral roots stained positively with monoclonal antibodies INO (*a*), C21 (*b*), C24 (*c*), and C17 (*d*); *e-h* are the phase micrographs of the fields seen in *a-d*, respectively. In the ventral root, all four antibodies bind specifically to the endoneurium surrounding axons, and in each case, staining is continuous with short fingers of the PNS that project into the spinal cord (arrows). However, except for blood vessels, staining is absent within the CNS proper. INO (*a*) alone also stained nuclei (presumably neuronal, see Fig. 10) within the spinal cord. Fields in *a* and *b* are taken from consecutive sections. Bar, 50 μ m.

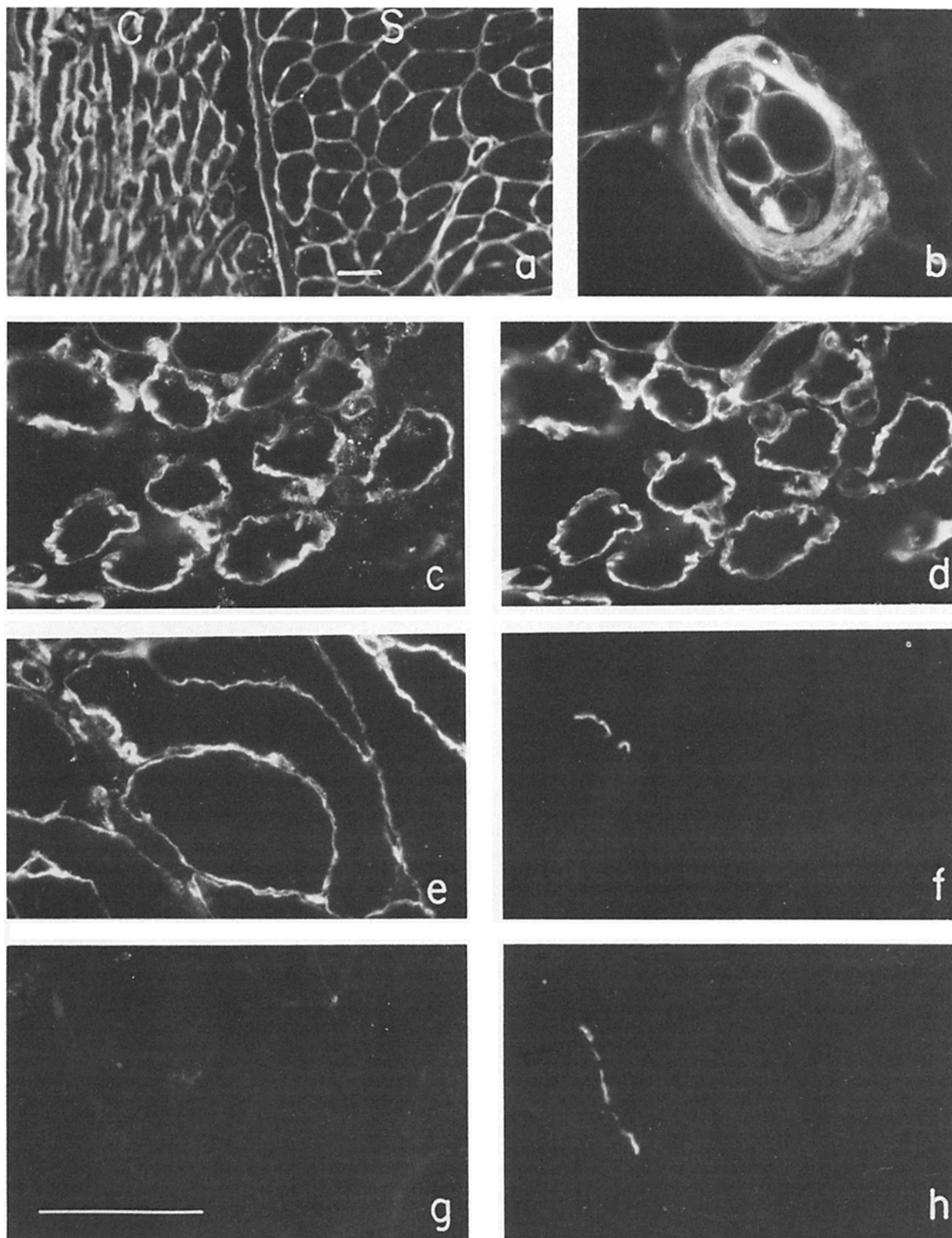


Figure 10. INO binds to the surfaces of several types of rat muscles including (a) cardiac (C) and skeletal (S) muscles, and (b) muscle spindles. INO immunoreactivity persists in empty basement membrane sheaths after muscle fibers were damaged and removed by phagocytosis (c). This section is double-labeled with rabbit anti-laminin and INO; INO-positive profiles co-localize with anti-laminin staining (d). At the neuromuscular junction, INO binding (e) is also present at postsynaptic sites concomitantly marked by rhodamine- α -bungarotoxin (f). In a control section, no immunoreactivity is seen when primary antibody was omitted (g); the endplate in this section is again marked by rhodamine- α -bungarotoxin (h). Magnification is identical for b-h; bars, 50 μ m.

antibodies to laminin and to HeSPG reveal that INO binds to basement membranes that also bear both laminin and HeSPG. This observation is consistent with the biochemical evidence that INO binding requires the presence of these two matrix molecules. Thus *in vivo*, as *in vitro*, the INO-binding site colocalizes with laminin and HeSPG in the extracellular matrix.

In situ localization of the INO epitope also shows that it is consistently found in locations known to support neuronal regeneration. Thus INO binding is detectable throughout the PNS, in the basal lamina of both myelinating and nonmyelinating Schwann cells; immunoreactivity is present where the PNS begins, in the transitional region in spinal roots, and extends to the most distal reaches of the periphery, including the terminal arbors of motoneurons. In marked contrast, no cell surface INO binding is seen in the adult rat CNS. Numerous studies have documented the ability of neurons to regenerate in the PNS after damage; new processes often grow long distances to re-establish synaptic contacts with target cells. Such extensive regeneration does not occur within the CNS where neurite outgrowth is soon aborted. This inability to regenerate is not intrinsic to neurons of the CNS, rather the extracellular milieu of the PNS appears to be much more supportive of axonal growth. CNS neurons can regenerate relatively long distances into a graft of peripheral nerve *in vivo* (5), or when provided with a suitable substrate *in vitro* (29, 31, 39). The segregation of the INO epitope in the rat nervous system suggests that one component of the environment that facilitates axonal outgrowth, namely a neurite-promoting extracellular matrix, is absent in the CNS, and may be a key reason for the inability of neurons to regenerate in the CNS.

It is also well known that denervated target cells provide signals that promote neuronal sprouting, even by uninjured neighboring axons (7, 13, 37). Such signaling can occur via several means. For example, diffusible sprouting factors may

be released (21) and cell surface molecules that render the target attractive to neurons may be produced (15). Our observation that the INO epitope is dramatically increased in the basement membrane surrounding muscles after denervation suggests a third means of promoting sprouting. Thus target cells may use several mechanisms simultaneously to attract neurons and hasten reinnervation. In denervated muscle, there is no apparent change in laminin; however, the rise in INO binding is accompanied by a detectable increase in HeSPG immunoreactivity. Furthermore, the intensity of staining by both INO and C17 increases with duration of denervation. Since the biochemical evidence indicates that INO binding requires both HeSPG and laminin, these observations suggest that in muscle basement membrane, the level of the INO epitope is limited by the availability of HeSPG.

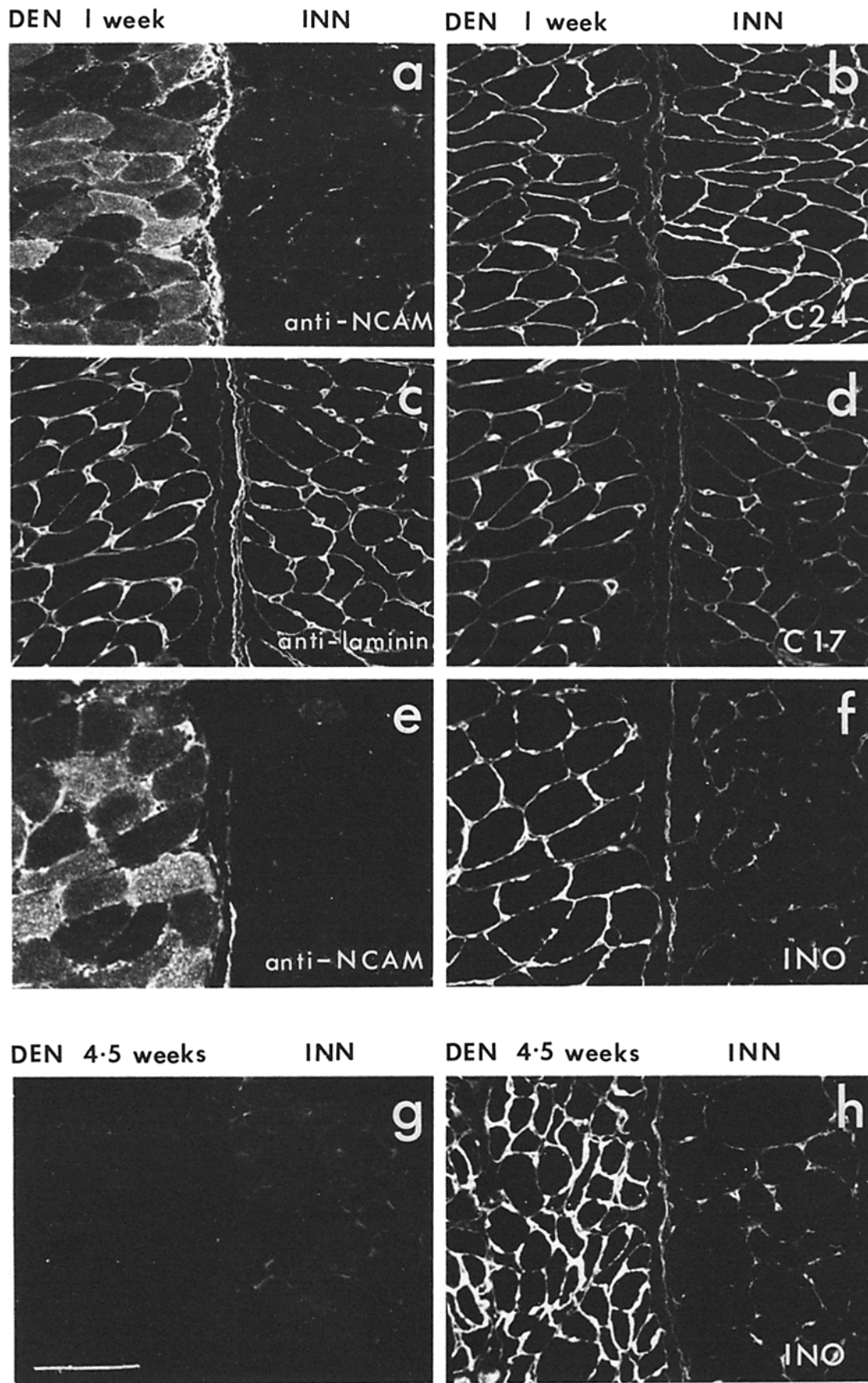
In summary, our reassociation studies show that formation of the HeSPG-laminin complex generates a new epitope, the INO-binding site, which plays an important role in neurite regeneration. Several lines of evidence support the significance of this epitope, and thus the whole complex, in neuronal regeneration: (a) INO, a monospecific reagent, can both precipitate and, more importantly, block the activity of the entire complex. (b) The INO epitope is localized both in the nervous system and in target tissues, at sites known to support or enhance axonal regeneration, but is not detected in areas where neurons do not normally regenerate or traverse. Laminin, on the other hand, has a much more widespread distribution; for example, it is abundantly expressed in the basement membrane-rich kidney where axons do not normally ramify. (c) In experiments designed to mimic the physiological substrates for regeneration, INO inhibits neuronal outgrowth on frozen sections of peripheral nerves, whereas anti-laminin does not (40). These results argue that the physiological substrate upon which axonal regeneration normally occurs is the HeSPG-laminin complex.

Table IV. Basement Membrane Staining

	C17 (HeSPG)	C24 (Laminin)	C21 (Laminin)	Laminin antisera	INO
Adrenal					
Cortex	+++	—	—	—	—
Medulla	+++	+++	+++	+++	++
Kidney					
Tubules	*—	++	++	++	—
Glomerular capsule	++	+/-	—	—	++
Glomerular matrix	++	++	—	—	—
Large blood vessels	+++	+++	—	+	—
Skeletal muscle endomysium					
Extrasynaptic	+++	+++	+++	+++	++
Synaptic	+++	+++	—	+++	++
Myotendinous junction	+++	+++	—	+++	++
Intramuscular nerve					
Perineurium	+++	+++	—	+++	+++
Endoneurium	+++	+++	+++	+++	++
Sciatic nerve and peripheral ganglia					
Epineurium	+++	+++	—	+++	—
Perineurium	+++	+++	—	+++	++
Endoneurium	+++	+++	+++	+++	++

Intensity of immunofluorescent staining range from extremely bright (+++), bright (++), faint (+), to undetectable (—).

* In other studies (18, 42), C17 has been known to stain tubules in frozen sections of Sprague-Dawley rat kidneys.



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Figure 11. Effect of denervation on INO immunoreactivity on skeletal muscles. 4- μ m sections of denervated rat hemidiaphragm, sandwiched against the innervated, contralateral hemidiaphragm, were stained with anti-N-CAM (a and e), C24 (b), anti-laminin (c), C17 (d), and INO (f and h), and without primary antibody (g). Muscles were removed 7 d postsurgery in a-f and after 4.5 wk in g and h. The sections stained with mAb's in b, f, and d were double-labeled with polyclonal rabbit antisera against N-CAM (a and e) and laminin (c), respectively. The presence of N-CAM provided direct confirmation of denervation. After 7 d of denervation, no difference in staining between experimental and control muscles is discernable with C24 (b) or anti-laminin (c), whereas a slight increase in C17 binding (d) is noted on denervated muscles in the same section. The greatest difference, however, between denervated and control muscles is found with INO staining (f). This difference increases with duration of denervation and is striking after 4.5 wk (h). In control sections (g), denervated muscles always show less background staining than innervated controls. Bar, 100 μ m.

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